Form PTO-1390 (REV 10-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
1581.0900000/RWE/MTT	
U S APPLICATION NO (IF KNOWN, SEE 37 C F R § 1 5)	
To be assigned 10/070764	
INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/03519	13 September 2000	13 September 1999

TITLE OF INVENTION

Preparation of Highly Pure Toxin Fragments

APPLICANT(S) FOR DO/EO/US

CHADDOCK, John Andrew, ALEXANDER, Frances Celine Gail, FOSTER, Keith Alan

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U S.C. 371
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S C 372(c)(3))
9. ☒ An oath or declaration of the inventor(s) (35 U.S C 371(c)(4))
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C F.R. 1 97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 C.F R. 3.28 and 3.31 is included
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    (a) Power of Attorney from Assignee with Delegation
    (b) Certificate Under 37 C.F.R. § 3.73(b)
    (c) Copy of International Published Application along with the International Search Report
    (d) Copy of the Notification of Transmittal of the International Preliminary Examination Report with attached International Preliminary Examination Report and annexes
    (e) Authorization to Treat a Reply as Incorporating an Extension of Time Under 37 C.F.R. § 1.136(a)(3)

JC10 Rec'd PCT/PTO 12 MAR 2002

U.S. APPLICATION NO (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
To be assigned 10/070764	PCT/GB00/03519	1581.0900000

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... **$ 100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		

Claims	Number Filed	Number Extra	Rate		
Total Claims	19- 20 =	0	X **$18.00**	$ 00 00	
Independent Claims	5- 3 =	2	X **$84.00**	$ 168.00	
Multiple dependent claim(s) (if applicable)			+ **$280.00**	$ 00.00	
TOTAL OF ABOVE CALCULATIONS =				$ 168.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$ 00 00	
SUBTOTAL =				$ 1058.00	
Processing fee of $130 00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				$ 00.00	
TOTAL NATIONAL FEE =				$ 1058 00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 40 00	
TOTAL FEES ENCLOSED =				$ 1098.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $ 1098.00 to cover the above fees is enclosed

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: *To be assigned*
CHADDOCK, J. A. *et al.*	Art Unit: *To be assigned*
Appl. No. (U.S. Natl. Phase of PCT/GB00/03519)	Examiner: *To be assigned*
Filed: (Int. Filing Date: September 13, 2000)	Atty. Docket: 1581.0900000/RWE/MTT
For: **Preparation of Highly Pure Toxin Fragments**	

Preliminary Amendment

Commissioner for Patents
Washington, D.C. 20231

Sir:

In advance of prosecution of the captioned application, Applicants submit the following Preliminary Amendments and Remarks. This Preliminary Amendment is provided in the following format:

(A) A clean version of each replacement paragraph/section/claim along with clear instructions for entry;

(B) Starting on a separate page, appropriate remarks and arguments. 37 C.F.R. § 1.115 and MPEP 714; and

(C) Starting on a separate page, a marked-up version entitled: "Version with markings to show changes made."

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of

this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Amendments

Please amend the application as follows:

In the Specification:

In the specification at page 1, before line 1, please insert the following paragraph:

The present application is a 371 of PCT/GB00/03519 filed on September 13, 2000, and published in English on March 22, 2001.

In the Abstract:

Please insert following abstract on page 23 after the claims:

Toxin derivatives are prepared by proteolytic treatment of holotoxin, and their toxicity is reduced by contacting the preparation with a ligand, which can be a metal or an antibody or another ligand. This ligand selectively binds to the toxin but not to the toxin derivative. Removing the ligand and toxin bound to the ligand further reduces toxicity. A second ligand is used to remove conjugates of the toxin and the first ligand. Compositions contain the purified derivative, optionally plus the toxin and the ligand.

This abstract is provided on a separate sheet appended hereto.

In the Claims:

Please amend the following claims from the currently pending claims as submitted under Article 34 to the International Bureau on December 26, 2001, and attached as annexes to the International Preliminary Examination Report:

Please cancel claims 1-21 without prejudice to or disclaimer of the subject matter contained therein. Applicants reserve the right to prosecute these claims in later continuing applications.

Please add the following new claims:

22. (New) A method of reducing toxicity of a clostridial toxin derivative preparation, comprising contacting said preparation with a ligand which selectively binds to the toxin but not to the toxin derivative.

23. (New) The method of claim 22, wherein the ligand binds to an H_C portion of the toxin.

24. (New) The method of claim 23, wherein the ligand is or comprises a metal ion which binds to the H_C portion of the toxin.

25. (New) The method of claim 22, wherein the ligand is an antibody that binds to the toxin.

26. (New) The method of claim 25, comprising contacting the preparation with a plurality of antibodies which selectively bind the toxin but not the toxin derivative.

27. (New) The method of claim 22, further comprising separating the ligand from the toxin derivative preparation.

28. (New) The method of claim 27, wherein the ligand is part of, or is bound to, or is otherwise attached to an affinity column.

29. (New) The method of claim 22, wherein the derivative is an LH_N fragment.

30. (New) The method of claim 22, wherein the derivative is a conjugate of an LH_N fragment with a targeting ligand.

31. (New) An affinity chromatography column, for removal of clostridial toxin from a clostridial toxin derivative preparation, wherein the column comprises a ligand that selectively binds to toxin but not to the clostridial toxin derivative.

32. (New) The column of claim 31, wherein the ligand is selected from the group consisting of an antibody and a toxin receptor.

33. (New) A clostridial toxin derivative preparation comprising 1-100 ppm clostridial toxin per toxin derivative.

34. (New) A composition comprising a derivative of a clostridial toxin and a pharmaceutically acceptable carrier, and further comprising a ligand that binds selectively to the toxin.

35. (New) The composition of claim 34, comprising a conjugate of a toxin with a ligand that binds selectively to the toxin, wherein the toxin is bound non-covalently to the ligand.

36. (New) The composition of claim 35, wherein the ligand is an antibody that selectively binds to the toxin.

37. (New) A method of removing a clostridial toxin from a preparation, said preparation containing the toxin and a derivative of the toxin, comprising:

contacting the preparation with a first ligand which binds to the clostridial toxin but not to the derivative;

separating the first ligand from the preparation to obtain a first treated preparation;

contacting the first treated preparation with a second ligand which binds to the first ligand but not to the derivative; and

separating the second ligand from the first treated preparation to obtain a second treated preparation.

38. (New) The method of claim 37, wherein the first ligand is attached to an affinity column.

39. (New) The method of claim 37, wherein the first ligand is an antibody.

40. (New) The method of claim 37, wherein the second ligand is an antibody.

Remarks

By the foregoing amendments, claims 1-21 have been cancelled and new claims 22-40 are sought to be entered. Support for the foregoing amendments to the claims may be found throughout the specification and in the claims as originally filed. Accordingly, the present amendments do not add new matter, and their entry is respectfully requested. Upon entry of the foregoing amendments, claims 22-40 are pending in the application, with claims 22, 31, 33, 34, and 37 being the independent claims.

It is believed that the present application is in condition for immediate examination. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Robert W. Esmond

Robert W. Esmond
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Date: March 12, 2002

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Version with markings to show changes made

In the Specification:

In the specification at page 1, before line 1, please insert the following paragraph:

The present application is a 371 of PCT/GB00/03519 filed on September 13, 2000, and published in English on March 22, 2001.

In the Abstract:

Please insert following abstract on page 23 after the claims:

Toxin derivatives are prepared by proteolytic treatment of holotoxin, and their toxicity is reduced by contacting the preparation with a ligand, which can be a metal or an antibody or another ligand. This ligand selectively binds to the toxin but not to the toxin derivative. Removing the ligand and toxin bound to the ligand further reduces toxicity. A second ligand is used to remove conjugates of the toxin and the first ligand. Compositions contain the purified derivative, optionally plus the toxin and the ligand.

In the Claims:

Claims 1-21 have been cancelled.

New claims 22-40 are sought to be entered.

PREPARATION OF HIGHLY PURE TOXIN FRAGMENTS

ABSTRACT

Toxin derivatives are prepared by proteolytic treatment of holotoxin, and their toxicity is reduced by contacting the preparation with a ligand, which can be a metal or an antibody or another ligand. This ligand selectively binds to the toxin but not to the toxin derivative. Removing the ligand and toxin bound to the ligand further reduces toxicity. A second ligand is used to remove conjugates of the toxin and the first ligand. Compositions contain the purified derivative, optionally plus the toxin and the ligand.

PREPARATION OF HIGHLY PURE TOXIN FRAGMENTS

This invention relates to derivatives, such as fragments, of toxins, particularly clostridial neurotoxins. It also relates to preparations containing those derivatives and to methods of obtaining the derivatives and the preparations.

The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of bacterium of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C_1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesised by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa.

The clostridial neurotoxins bind to an acceptor site (Black, J. D. & Dolly, J. O., Neuroscience, **23**, 767-779, 1987 and Dolly *et al.* in Cellular and Molecular Basis of Cholinergic Function, ed. Dowdall, M. J. & Hawthorne, J. N., Chapter 60, 1987) on the cell membrane of the motoneurone at the neuromuscular junction and are internalised by an endocytotic mechanism (Montecucco *et al.*, Trends Biochem. Sci., **11**, 314, 1986). It is believed that the clostridial neurotoxins are highly selective for motoneurons due to the specific nature of the acceptor site on those neurones. The binding activity of clostridial neurotoxins is known to reside in a carboxy-terminal region of the heavy chain component of the dichain neurotoxin molecule, a region known as H_C. The N-terminal region of the H-chain (H_N domain) is thought to be of central importance in the translocation of the L-chain

into the cytosol and has been demonstrated to from channels in lipid vesicles (Shone *et al*, Eur. J. Biochem. **167**, 175-180).

Clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity that is known to reside in the L-chain. Each toxin serotype hydrolyses a specific peptide bond within one of three proteins of the SNARE complex; VAMP (synaptobrevin), syntaxin or SNAP-25. Proteolytic cleavage of one of these proteins leads to instability of the SNARE complex and consequent prevention of vesicular release. The enzymatic activity of the light chain of the neurotoxin leads to inhibition of neurotransmitter release, which results in a prolonged muscular paralysis.

The central role of the SNARE proteins in regulated secretion has been convincingly established (e.g. Niemann *et al.*, (1994) *Trends Cell Biol.*, 4, 179-185). However, the correlation of SNARE protein involvement with the release of specific hormones, peptides, transmitters and other signalling molecules remains to be established in the majority of cases. The range of highly specific endopeptidase activities of clostridial neurotoxin serotypes provides a unique approach to the understanding of SNARE-mediated events. Unfortunately, the use of native clostridial toxins for the study of such events is limited by at least two important aspects. Firstly, the expression of the requisite toxin receptor is restricted to a limited population of cells, thereby limiting the range of cell types in which SNARE-mediated events can be studied without cellular disruption. Secondly, the significant hazards associated with working with potent neurotoxins lead to restrictions on the range of applications and experimental design. Clostridial neurotoxins are the most potent neuroparalytic toxins known and must be manipulated in specialised laboratory conditions by specially trained and, preferably, vaccinated staff. The ability to produce highly purified non-toxic fragments of clostridial neurotoxins possessing the enzymatic activity of the clostridial neurotoxins and capable of delivery to the cytosol of selected cells would therefore provide a valuable tool for

studying secretory mechanisms.

The ability of the clostridial neurotoxins' enzymatic activity to destabilise SNARE complex formation and thereby inhibit vesicle fusion at the plasma membrane also has therapeutic potential. A number of therapeutic applications have been proposed (e.g. WO 96/33273 & WO 94/21300) that are dependent on the successful retargeting of clostridial neurotoxin fragments. These approaches require a source of non-hazardous neurotoxin fragment that is suitable for the synthesis of non-toxic conjugates, since the side effect profile of a therapeutic contaminated with neurotoxin would be unacceptably high. In addition to retargeting of clostridial toxin fragments, there are further applications for non-toxic clostridial derivatives. For example, as an immunogen for vaccine preparation, as a source of material from which highly purified neurotoxin-related fragments can be prepared, and as a non-toxic endopeptidase standard in diagnostic kits (e.g. WO 95/33850).

Since the cell binding function of clostridial neurotoxins resides in the H_C domain of the heavy chain, generation of a fragment in which the binding capability of the H_C has been deleted but the properties of the H_N domain are retained (LH_N) is potentially a suitable method for the production of a non-toxic derivative.

It is known to prepare these fragments by proteolytic treatment of toxin and then separation of toxin from fragments by anion exchange chromatography, and such methods successfully yield fragments that are 99.99% pure.

A central or recurrent problem associated with obtaining or using products from toxins made by these methods is the risk of residual toxicity in those products. It would hence be desirable to provide a method of removing toxin from such products. However, existing protocols have reached the limits of their abilities in this respect.

For example, it has been observed that the known fragments often exhibit a high inhibition of neurotransmitter release by neuronal cells *in vitro*. This has hampered investigation into the properties of conjugates in which a toxin fragment is combined with a ligand providing a specific targeting function, because of difficulty in providing controls against which to judge the conjugate activity.

It is an object of the present invention to provide a method of preparing a toxin derivative preparation. A further object of the invention is to provide a method of removing toxin from a toxin derivative preparation.

It has been discovered in accordance with the present invention that existing toxin derivative preparations, though considered to be pure, and though containing toxin at an extremely low level, nevertheless contain sufficient residual toxin to interfere with the applications of the fragment, conjugate or other toxin derivative.

Thus, in a first aspect of the invention, there is provided a method of reducing toxicity of a toxin derivative preparation, comprising contacting said preparation with a ligand which selectively binds to the toxin but not to the toxin derivative.

In a use of the invention, the ligand binds to and effectively neutralises residual toxin which is contaminating the toxin derivative preparation. The ligand preferentially binds to the toxin compared with its binding to the

derivative. Thus in use there may be some small loss of derivative at the same time as toxin binds to the ligand. It has been found that prior art preparations of toxin derivatives may contain toxin at levels of one toxin molecule per 10,000 toxin fragments, and even at this low level the toxin adversely effects the uses of the fragment. According to the invention, an antibody that preferentially binds to the toxin, but not to the derivative, can be used to reduce or remove toxicity associated with the toxin, thereby enabling the effects and applications of the derivative to be examined and used without any masking effect of the residual toxin.

In an embodiment of the invention, described in more detail below, a toxin fragment prepared according to prior art methods significantly inhibited substance P release from dorsal root ganglia. This inhibition was reduced almost entirely by the combination of the fragment with an antibody that specifically bound to toxin.

Antibodies for use in the present invention can be prepared using polyclonal or monoclonal techniques. Suitable methodology is found for example in "Antibodies: A laboratory manual, by Ed Harlow and David Lane, 1988". Monoclonal antibodies can be prepared by immunising mice against toxin, harvesting lymphocyte cells from the spleens of immunised mice and fusing these with myeloma cells. Antibodies secreted by the resulting hybridomas are screened for binding to toxin and positive clones selected and propagated. Monoclonal antibodies are harvested from cultured hybridomas and purified using chromatographic methods - see for example Pharmacia handbook on "Monoclonal antibody purification". As an alternative to immunising mice with the toxin itself, the mice can be immunised with a different source of a H_c domain, whether obtained from native material or expressed in an alternative, non clostridial, host. Alternatively, mice can be immunised with toxoided preparations of intact neurotoxins and the anti Hc antibodies selected. This may be achieved in at least 2 ways for example: specific Hc antisera may be bound to

immobilised Hc and subsequently eluted for use. Conversely, specific Hc antisera may be obtained by adsorbing non-Hc antisera onto LH_N.

This invention thus provides, in specific examples, preparation of specific, defined fragments from native clostridial neurotoxins. Purified clostridial neurotoxin obtained from *Clostridia sp.* using previously reported techniques (for example, Shone, C. C. and Tranter, H. S. (1995) *in* "Clostridial Neurotoxins - The molecular pathogenesis of tetanus and botulism", (Montecucco, C., Ed.), pp. 152-160, Springer) can be fragmented by proteolytic or chemical cleavage to yield a crude mixture of derivatives that possess elements of the light chain, heavy chain or both (Gimenez, J. A. & DasGupta, B. R. (1993) J. Protein Chem., **12**, 351-363; Shone, C. C., Hambleton, P. and Melling, J. (1987) *Eur. J. Biochem.* **167**, 175-180). Classical chromatographic techniques are used to separate the crude mixture into partially purified fragments, the residual toxicity of which would make them unsuitable for many applications, and the fragments are then combined with a neutralizing ligand.

During testing by the inventors of a conjugate or a toxin fragment with a targeting ligand, it was not possible *in vitro* to determine the selectivity of an LH_N fragment, though the reasons for this were not known to the inventors at the time. According to the invention, it has surprisingly been found that very low levels of toxins present were masking the LH_N fragment activity, and now advantageously it is possible further to reduce this toxin content so that pure LH_N activity can be measured and assessed.

In the particular case of *in vivo* uses of fragments and conjugates and other derivatives, a specific embodiment of the invention, described in more detail below, determined that fragments according to the present invention exhibited a level of toxicity that was more than 10 times lower than that of the prior art fragments, which prior art fragments were hitherto considered as being pure and toxin-free.

It is a preferred additional step in the method of the invention to separate the ligand from the toxin derivative preparation.

In one embodiment of the invention, a specific binding event between an immobilised matrix and a domain present on the neurotoxin, but absent from the fragment, is used to separate neurotoxin from the fragment. Such a method is used specifically to bind neurotoxin and other fragments possessing the requisite binding domain from a crude neurotoxin fragment mixture. Non-binding fragments that are free of neurotoxin are simply isolated from the column flow through. Binding fragments including neurotoxin can be isolated by altering the conditions of binding, for instance by altering the chemical environment (e.g. pH, ionic strength) or incorporation of a substance that competes for the binding site (e.g. peptide, sugar moiety). One suitable example is a column containing the natural receptor, or a version of the receptor, for which the neurotoxin has an affinity. This receptor may be purified and immobilised to a matrix for use in a column or free in solution, or present in a preparation of cells or cell membrane, and is of use for purification of LH_N. Typically, a crude preparation of clostridial neurotoxin fragments is applied to the receptor preparation to bind neurotoxin and other fragments that possess receptor binding properties. Non-binding fragments, which include LH_N, are recovered from the receptor preparation by simple elution. Binding fragments and neurotoxin are released and harvested as described above.

Purification of clostridial endopeptidases is also suitably achieved according to the invention using metal ion chromatography. Clostridial endopeptidases are characterised as metalloendopeptidases due to the co-ordination of a metal ion at the active site of the enzyme. Given this specific metal ion binding capacity of clostridial neurotoxins, it might be predicted that both neurotoxin and endopeptidase fragments bind to immobilised metal resin via this catalytic site interaction. However, it is found that the LH_N does not bind to the chelating column whereas the

neurotoxin does. In an example, set out in more detail below, a method for the purification of LH_N/A utilises immobilised zinc ions to bind BoNT/A and purify LH_N/A. The low toxicity of LH_N/A purified by this method is also confirmed below.

Another embodiment of the invention comprises immobilising specific antibodies to a column resin. The antibodies are selected on the basis of their specificity for epitopes present on the neurotoxin but absent on the desired fragment, exemplified by LH_N below. In the presence of partially purified LH_N, antibodies with specificity for the H_C domain will only bind to the neurotoxin. Contaminating neurotoxin is removed from the LH_N preparation by entrapment on the immobilised-antibody matrix, whereas LH_N, which is not recognised by the antibodies, does not interact with the column. This method is surprisingly efficient at removing residual toxicity from the LH_N preparation and affords an effective purification technique. Entrapment of the neurotoxin contaminant by antibody binding, rather than specifically binding the LH_N, enables the elution conditions to be maintained at the optimum for LH_N stability. The use of harsh elution conditions e.g. low pH, high salt, chaotropic ions, which may have detrimental effects on LH_N polypeptide folding and enzymatic activity, are therefore avoided. Neurotoxin and other binding fragments may be eluted from the antibody column to release clostridial neurotoxin derivatives that are purified from derivatives deficient in the H_C domain epitopes.

An additional advantage of the methods of the invention is thus that the desired component of the mixture that is being purified, that is to say the fragment or the conjugate or the other derivative, is that portion which is eluted from the column whereas the undesired portion, the toxin, remains bound to the column. This has the benefit that the desired material is less affected by the column and that no additional step, for example to elute bound, desired material from the column, is required as part of the method.

Further embodiments of the invention use two different affinity techniques in combination. In a preferred embodiment of the invention exemplified below, combinations of antibodies with different epitope recognition properties are used. By utilising monoclonal antibodies with different recognition epitopes, small conformational changes in neurotoxin can be accommodated. In this way, a greater proportion of neurotoxin is targeted for removal from the crude starting mixture.

It is further preferred that the method of the invention comprises an additional step, after separating the ligand from the toxin derivative preparation, which ligand we will refer to in these present paragraphs as the first ligand, of contacting the toxin derivative preparation with a second ligand, which selectively binds to the first ligand but not to the toxin derivative. It is occasionally the case that ligand attached, for example, to a chromatography column, detaches and thus the toxin derivative elutes from the column in combination with complexes of ligand and toxin. This opens the possibility of separation of the complex at a future time, releasing the toxin. It is an advantage of the preferred method of the invention that these ligand-toxin complexes, if present in the toxin derivative preparation are substantially removed by use of the further ligand.

The further, or second, ligand can suitably be an antibody that binds to the antibody used as the first ligand. A specific embodiment of the invention thus comprises:-

preparing a toxin derivative preparation which comprises toxin derivative which is contaminated by low levels of toxin;

contacting the preparation with a ligand which selectively binds to the toxin but not to the toxin derivative;

separating the ligand from the toxin derivative preparation, thereby separating toxin from the toxin derivative preparation;

contacting the preparation with a further ligand that binds selectively to the first ligand, or binds selectively to a complex of the first ligand with toxin; and

separating the second ligand from the toxin derivative preparation.

The first ligand can be an antibody which binds to an H_c portion of the toxin, and the second ligand can be a further antibody or immunoglobulin binding domain which binds to the first antibody or to a complex of the first antibody with toxin, and in a specific example the second ligand can be protein G. In one particular example of the preferred embodiment of the invention in use, injection of 20 micrograms LH_N purified according to the invention using the first ligand resulted in 0 out of 4 mice surviving, whereas use of the second ligand to remove antibody - toxin complexes resulted in 4 out of 4 survivors. This very surprising result exhibits the improved purity of the toxin derivative following application of the second purification step.

Specific antibodies or antibody fragments are optionally mixed with partially purified clostridial neurotoxin fragments in solution to form bound antibody-neurotoxin complexes. The antibody-neurotoxin complexes are then isolated from the mixture (e.g. by Protein G chromatography) and removed to yield purified agents in solution.

Immobilised monoclonal antibodies may be used specifically to bind contaminating neurotoxin and intact H_c or other contaminants. Two BoNT/A H_c specific monoclonal antibodies are thus utilised in a method that is surprisingly efficient at binding contaminating neurotoxin to prepare LH_N/A of high purity and low toxicity.

The present invention is of application to any toxins which can be converted into useful fragments, conjugates and other derivatives, for example by proteolytic action, and is of particular application to clostridial neurotoxins, especially botulinum and tetanus toxins of all sub-groups.

By toxin derivative, it is intended to encompass all derivatives of a toxin that are prepared directly or indirectly from native toxin, for example by proteolytic action on the toxin, which methods can result in a preparation having residual, low levels of toxin present. Thus, a toxin derivative preparation according to the definition does not include recombinant toxin derivatives which are guaranteed to be free of toxin. The meaning of derivative thus encompasses toxin fragments and conjugates of toxin fragments with other molecules as well as variants of toxins and toxin fragments and conjugates.

In a further aspect, the invention provides an affinity chromatography column, for removal of toxin from a toxin derivative preparation, wherein the column comprises a ligand that selectively binds to toxin but not to the toxin derivative. This column is of use in separating toxin from toxin derivative, and the ligand employed is suitably an antibody or a metal ion.

A still further aspect of the invention lies in a toxin derivative preparation comprising 1-100 ppm toxin per toxin derivative, preferably 10 - 100ppm. This preparation is derived from native toxin, and has a greatly reduced residual level of toxin.

A yet further aspect of the invention provides a composition comprising a derivative of a toxin and a pharmaceutically acceptable carrier, and further comprising a ligand that binds selectively to the toxin. The composition may for example comprise a conjugate of a toxin with a ligand that binds selectively to the toxin, wherein the toxin is bound non-covalently to the ligand. The toxin is thus neutralized by the ligand.

The invention is now described in specific embodiments, accompanied by drawings in which:-

Fig. 1 shows SDS-PAGE analysis of LH_N/A purified by the immunoaffinity approach;

Fig. 2 shows retention of functional activity of LH_N/A prepared by the invention

Fig. 3 shows inhibition of neurotransmitter release from eSCN by LH_N/A and BoNT/A; and

Fig. 4 shows mouse toxicity data for purified LH_N/A.

Example 1

Inhibition of Glycine Release

Embryonic spinal cord cells were treated for one day in the presence of 30 micrograms per ml of LH_N/A (prior art preparation). This treatment resulted in an inhibition of glycine release of 64%. A parallel treatment was carried out in the presence of antibody 5BA 9.3, which binds specifically to botulinum neurotoxin A. This resulted in an inhibition of glycine release which was reduced to 44%.

Example 2

Inhibition of Substance P Release

Embryonic Dorsal route ganglia were treated for 3 days with 20 micrograms per ml LH_N/A, LH_N/A + antibody 5BA 9.3 and also with a conjugate of LH_N/A with a targeting ligand in the presence of antibody 5BA 9.3.

The inhibition of substance P release was significant when the 20μg/ml

LH_N/A fragment was used alone, and was at a level of about 26%. This level was reduced to about 4% in the presence of the specific antibody, and the level rose to about 21% when the conjugate (also 20μg/ml) was used in the presence of the specific antibody.

Example 3

Production of LH_N/A from BoNT/A by antibody-affinity chromatography

BoNT/A was prepared according to a previous method (Shone, C. C. and Tranter, H. S. (1995) *in* "Clostridial Neurotoxins - The molecular pathogenesis of tetanus and botulism", (Montecucco, C., Ed.), pp. 152-160, Springer). FPLC® chromatography media and columns were obtained from Amersham Pharmacia Biotech, UK. Affi-gel Hz™ matrix and materials were from BioRad, UK.

Preparation of an anti-BoNT/A antibody-affinity column

An antibody-affinity column was prepared with specific monoclonal antibodies essentially as suggested by the manufacturers' protocol. Briefly, monoclonal antibodies 5BA2.3 & 5BA9.3 which have different epitope recognition in the H_C domain (Hallis, B., Fooks, S., Shone, C. and Hambleton, P. (1993) *in* "Botulinum and Tetanus Neurotoxins", (DasGupta, B. R., Ed.), pp. 433-436, Plenum Press, New York) were purified from mouse hybridoma tissue culture supernatant by Protein G (Amersham Pharmacia Biotech) chromatography. These antibodies represent a source of BoNT/A H_C-specific binding molecules and can be immobilised to a matrix or used free in solution to bind BoNT/A. In the presence of partially purified LH_N/A (which has no H_C domain) these antibodies will only bind to BoNT/A. The antibodies 5BA2.3 & 5BA9.3 were pooled in a 3:1 ratio and two mg of the pooled antibody was oxidised by the addition of sodium periodate (final concentration of 0.2%) prior coupling to 1ml Affi-Gel Hz™ gel (16 hours at room temperature). Coupling efficiencies were routinely

greater than 65%. The matrix was stored at 4°C in the presence of 0.02% sodium azide.

Purification strategy for the preparation of pure LH_N/A

BoNT/A was treated with 17μg trypsin per mg BoNT/A for a period of 72-120 hours. After this time no material of 150kDa was observed by SDS-PAGE and Coomassie blue staining. The trypsin digested sample was chromatographed (FPLC® system, Amersham Pharmacia Biotech) on a Mono Q® column (HR5/5) to remove trypsin and separate the majority of BoNT/A from LH_N/A. The crude sample was loaded onto the column at pH 7 in 20mM HEPES, 50mM NaCl and 2ml LH_N/A fractions eluted in a NaCl gradient from 50mM to 150mM. The slightly greater pI of BoNT/A (6.3) relative to LH_N/A (5.2) encouraged any BoNT/A remaining after trypsinisation to elute from the anion exchange column at a lower salt concentration than LH_N/A. LH_N/A containing fractions (as identified by SDS-PAGE) were pooled for application to the antibody column.

The semi-purified LH_N/A mixture was applied and reapplied at least 3 times to a 1-2ml immobilised monoclonal antibody matrix at 20°C. After a total of 3 hours in contact with the immobilised antibodies, the LH_N/A-enriched supernatant was removed. Entrapment of the BoNT/A contaminant, rather than specifically binding the LH_N/A, enables the elution conditions to be maintained at the optimum for LH_N stability. The use of harsh elution conditions e.g. low pH, high salt, chaotropic ions, which may have detrimental effects on LH_N polypeptide folding and enzymatic activity, are therefore avoided. Treatment of the immobilised antibody column with 0.2M glycine/HCl pH2.5 resulted in regeneration of the column and elution of BoNT/A-reactive proteins of 150kDa.

The LH_N/A enriched sample was then applied 2 times to a 1ml HiTrap® Protein G column (Amersham Pharmacia Biotech) at 20°C. Protein G was

selected since it has a high affinity for mouse monoclonal antibodies. This step was included to remove BoNT/A-antibody complexes that may leach from the immunocolumn. Antibody species bind to the Protein G matrix allowing purified LH_N/A to elute.

The profile of the purification procedure is illustrated by the SDS-PAGE analysis in Figure 1. Trypsin digested BoNT/A (lane 4) was applied to a Mono Q® anion exchange column and fractions harvested (lane 5). Material pre- and post-Protein G is indicated in lanes 6 and 7 respectively. Lane 7 represents the final purified LH_N/A preparation. Samples were analysed by SDS-PAGE on 4-20% polyacrylamide and stained with Coomassie blue (Panel A), or Western blotted and probed with anti-BoNT/A (Panel B). Molecular weight markers are indicated on the Figure with reference to the standards in lanes 2 & 8. Molecular weight markers in lane 1 are compatible with enhanced chemiluminescence and are for visualisation purposes only.

In vitro SNAP-25 peptide cleavage

The *in vitro* cleavage of SNAP-25 by LH_N/A and other endopeptidase samples were assessed essentially as described previously (Hallis, B., James, B. A. F. and Shone, C. C. (1996) *J. Clin. Microbiol.* **34**, 1934-1938). Figure 2 clearly demonstrates the similarity in catalytic activity between purified LH_N/A and reduced BoNT/A. A series of LH_N/A (●) and BoNT/A (□) dilutions were incubated for 1 hour prior to assessment of SNAP-25 cleavage. The data is representative of at least 7 experiments which have suggested EC_{50} for LH_N/A and BoNT/A to be 3.8 ± 0.7pM and 3.6 ± 0.6pM respectively.

SDS-PAGE and Western Blotting

SDS-PAGE and Western Blotting were performed using standard protocols.

Proteins were resolved on a 4-20% Tris/glycine polyacrylamide gel (Novex) and either stained by the addition of Coomassie blue or transferred to nitrocellulose. Positive binding of antibodies was detected by enhanced chemiluminescence.

Example 4

Production of LH_N/A from BoNT/A by immobilised metal affinity chromatography

BoNT/A was prepared according to a previous method (Shone, C. C. and Tranter, H. S. (1995) *in* "Clostridial Neurotoxins - The molecular pathogenesis of tetanus and botulism", (Montecucco, C., Ed.), pp. 152-160, Springer). FPLC® chromatography media and columns were obtained from Amersham Pharmacia Biotech, UK. Affi-gel Hz™ matrix and materials were from BioRad, UK.

Preparation of cationic metal affinity column

An affinity column was prepared essentially as suggested by the manufacturers' protocol. Briefly, chelating Sepharose® (Amersham Pharmacia Biotech) was washed with 50mM EDTA + 1M NaCl (10 column volumes), washed with 10 column volumes ultra-high purity water, primed with 5mg/ml $ZnCl_2$ (10 column volumes –neutral pH, filtered), and finally washed with purified water (10x column volumes).

Purification strategy for the preparation of pure LH_N/A

BoNT/A was treated with 17μg trypsin per mg BoNT/A for a period of 72-120 hours. After this time no material of 150kDa was observed by SDS-PAGE and Coomassie blue staining. The trypsin digested sample was chromatographed (FPLC® system, Amersham Pharmacia Biotech) on a Mono Q® column (HR5/5) to remove trypsin and separate the majority of BoNT/A

from LH_N/A. The crude sample was loaded onto the column at pH 7 in 20mM HEPES, 50mM NaCl and 2ml LH_N/A fractions eluted in a NaCl gradient from 50mM to 150mM. The slightly greater pI of BoNT/A (6.3) relative to LH_N/A (5.2) encouraged any BoNT/A remaining after trypsinisation to elute from the anion exchange column at a lower salt concentration than LH_N/A. LH_N/A containing fractions (as identified by SDS-PAGE) were pooled for application to the metal affinity column.

For the purification of 1mg LH_N/A a 1 ml column of Zn^{2+} primed chelating Sepharose®, prepared as described above was equilibrated to the appropriate pH (7.5-8.5) using 50mM HEPES + 1M NaCl ('equilibration buffer'). 1 mg of LH_N (post Mono Q® fractionation) was applied to the column after dialysis against the appropriate equilibration buffer. Material that did not bind was recovered and the column washed with excess equilibration buffer. Bound material was eluted by the application of equilibration buffer supplemented with 10mM EDTA.

Another suitable method is described by Rossetto et al, Biochem J., (1992), vol. 285, pp 9-12.

Example 5

Evaluation of BoNT/A contamination levels in LH_N/A prepared by immunocolumn chromatography in primary cultures of spinal cord neurons

Inhibition of [^{3}H]-glycine release from eSCN

Spinal cord neurons are exquisitely sensitive to neurotoxin, with an IC_{50} of inhibition of glycine release of 0.027 ± 0.0006pM after 3 days exposure to BoNT/A (unpublished observations). Therefore they serve as a suitably sensitive assay for screening samples for the presence of neurotoxin. Spinal cords dissected from 14-15 day old foetal Sprague Dawley rats were cultured for 21 days using a modification of previously described method

(Ransom, B. R., Neale, E., Henkart, M., Bullock, P. N. and Nelson, P. G. (1977) *J. Neurophysiol.* 40, 1132-1150 & Fitzgerald, S. C. (1989) "A dissection and tissue culture manual of the nervous system", Alan R. Liss, Inc., New York, NY). Cells were loaded with [^{3}H]-glycine for 30 minutes prior to determination of basal and potassium-stimulated release of transmitter (essentially as described in Williamson, L. C., Halpern, J. L., Montecucco, C., Brown, J. E. and Neale, E. A. (1996) *J. Biol. Chem.* **271**, 7694-7699). A sample of 0.2M NaOH-lysed cells was used to determine total counts, from which % release could be calculated.

In figure 3 there is shown a comparison of the inhibition curves obtained after 3 days incubation of spinal cord neurons with LH_N/A and BoNT/A. Samples of LH_N/A (●) or BoNT/A (□) were diluted in eSCN growth medium and 1ml of the appropriate concentration applied. Cells were incubated for three days prior to assessment of [^{3}H]-glycine release. The % inhibition data is calculated by relating the net stimulated [^{3}H]-glycine release to the total uptake and then expressing this as a percentage of the release obtained from control media treated cells. The data are representative of at least three experiments. The IC_{50} data determined for LH_N/A (106.2 ± 49.3nM) and BoNT/A can be used to estimate the ratio of BoNT/A to LH_N/A in the purified material. It is estimated that a maximum of 1 BoNT/A molecule per $4x10^6$ LH_N/A molecules was present in the final purified material.

Example 6

Removal of BoNT/A from LH_N/A preparations as assessed by mouse lethality

Residual BoNT/A contamination was evaluated following intraperitoneal injection of 0.5ml of test sample in gelatine-phosphate buffer (1% (w/v) Na_2HPO_4, 0.2% (w/v) gelatine, pH 6.5-6.6) into mice. After 4 days the number of surviving animals was counted. Literature precedents (Shone

et al. 1987 & de Pavia, A. and Dolly, J. O. 1990) cite the LD_{50} to be the concentration of test sample that killed half the animals in the test group within 4 days. By this analysis, batches of purified LH_N/A were demonstrated to exhibit an LD_{50} of approximately 50μg/mouse i.e. approximately 20LD_{50}/mg. This is significantly lower than previously reported LD_{50} data for LH_N/A (6000-12000LD_{50}/mg by Shone, C. C., Hambleton, P. and Melling, J. (1987) *Eur. J. Biochem.* **167**, 175-180) and LC/A (< 100LD_{50}/mg in Shone *et al.* 1987 & 10000LD_{50}/mg in de Pavia, A. and Dolly, J. O. (1990) *FEBS Lett.*, **277**, 171-174).

As an alternative method of data analysis, the mean number of survivors from different batches of LH_N/A was determined and is presented in Figure 4 (Abbreviations: IMAC; immobilised metal affinity column, ND; Not determined). These data clearly demonstrate the significantly low toxicity of the LH_N/A prepared by both exemplified methods of purification.

ART 34 AMDT

CLAIMS

1. A method of reducing toxicity of a clostridial toxin derivative preparation, comprising contacting said preparation with a first ligand which selectively binds to the toxin but not to the toxin derivative.

2. A method according to Claim 1 wherein the first ligand binds to an H_c portion of the toxin.

3. A method according to Claim 2 wherein the first ligand is or comprises a metal ion which binds to the H_c portion of the toxin, or is an antibody that binds to the toxin.

4. A method according to Claim 3 comprising contacting the preparation with a plurality of antibodies which selectively bind the toxin but not the toxin derivative.

5. A method of removing toxin from a toxin derivative preparation comprising contacting the preparation with a first ligand according to any of Claims 1 to 4 and further comprising separating the ligand from the toxin derivative preparation to obtain a treated preparation.

6. A method according to Claim 5 comprising contacting the treated preparation with a second ligand, which second ligand selectively binds to the first ligand but not to the toxin derivative, and separating the second ligand from the treated preparation.

7. A method according to Claim 5 or 6 wherein the first ligand is part of or is bound to or is otherwise attached to an affinity column.

8. A method according to Claim 6 or 7 wherein the second ligand is part of or is bound to or is otherwise attached to an affinity column.

9. A method according to any of Claims 1 to 8 wherein the toxin derivative is selected from a non-toxic fragment or variant of a toxin, a non-toxic conjugate comprising a fragment or a variant of a toxin and another derivative of a toxin which is obtained directly or indirectly from native toxin.

10. A method according to Claim 9 wherein the derivative is an LH_N fragment.

11. A method according to Claim 9 wherein the derivative is a conjugate of an LH_N fragment with a targeting ligand.

12. A method according to any of Claims 1 to 11, comprising obtaining the toxin derivative by cleavage of native toxin to yield a mixture of uncleaved toxin and toxin derivative, and subjecting that mixture to a purification step to remove uncleaved toxin.

13. A method according to Claim 12 comprising purifying the mixture so as to remove uncleaved toxin by anion - exchange chromatography, cation-exchange chromatography, hydrophobic interaction chromatography or size-exclusion chromatography.

14. An affinity chromatography column, for removal of toxin from a clostridial toxin derivative preparation, wherein the column comprises a first ligand that selectively binds to toxin but not to the toxin derivative.

15. A column according to Claim 14 wherein the ligand is selected from an antibody, and a toxin receptor.

16. A clostridial toxin derivative preparation comprising 1-100 ppm toxin per toxin derivative.

17. A composition comprising a derivative of a clostridial toxin and a pharmaceutically acceptable carrier, and further comprising a ligand that binds selectively to the toxin.

18. A composition according to Claim 17, comprising a conjugate of a toxin with a ligand that binds selectively to the toxin, wherein the toxin is bound non-covalently to the ligand.

19. A composition according to Claim 18 wherein the ligand is an antibody that selectively binds to the toxin.

20. A pharmaceutical composition comprising a clostridial toxin derivative or a composition according to any of Claims 16 - 19 in combination with a pharmaceutically acceptable carrier.

21. Use of an affinity chromatography column according to Claim 14 or 15 in combination with an affinity column comprising a second ligand that selectively binds the first ligand but not the toxin derivative for removal of toxin from a clostridial toxin derivative preparation.

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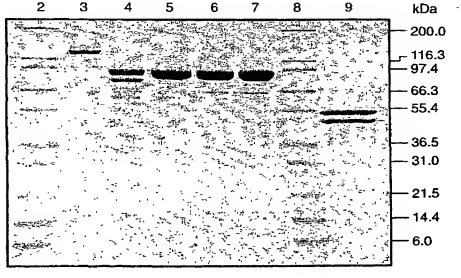
[Continued on next page]

(54) Title: PREPARATION OF HIGHLY PURE TOXIN FRAGMENTS

SDS-PAGE analysis of LH_N/A purified by immunoaffinity approach



(57) Abstract: Toxin derivatives are made by proteolytic treatment of holotoxin, and the toxicity of a toxin derivative preparation is reduced by contacting the preparation with a ligand, which can be a metal or an antibody or another ligand. This ligand selectively binds to the toxin but not to the toxin derivative. Removing the ligand and toxin bound to the ligand further reduces toxicity. Compositions containing the purified derivative, optionally plus the toxin and the ligand are described.

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FIG. 1

SDS-PAGE analysis of LH_N/A purified by immunoaffinity approach

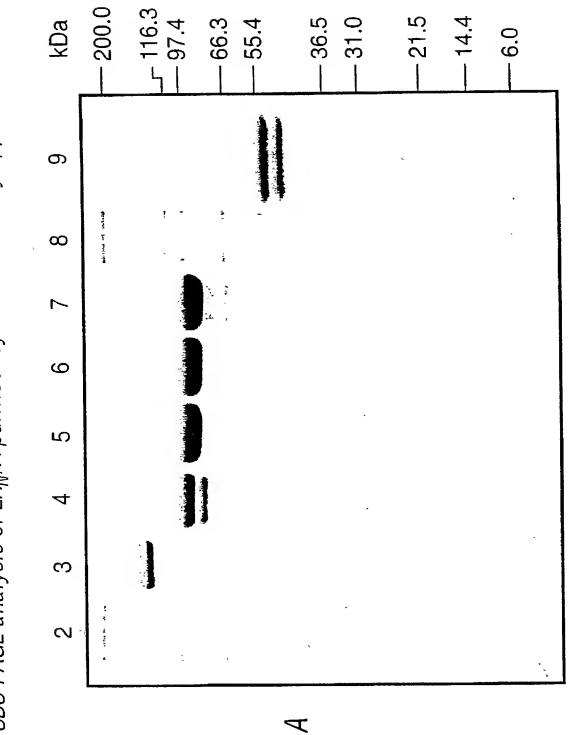

FIG. 1(contd.)

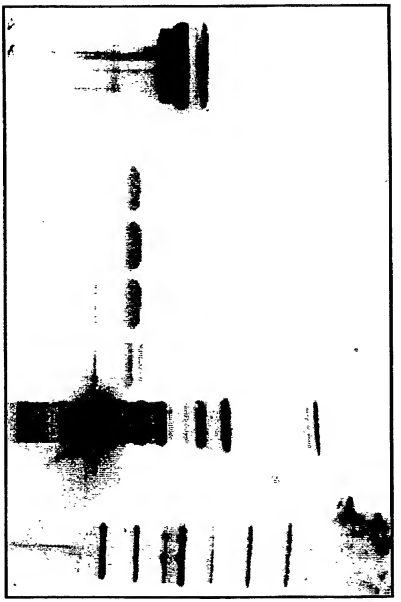

FIG. 2

Cleavage of SNAP-25 by LH_N/A

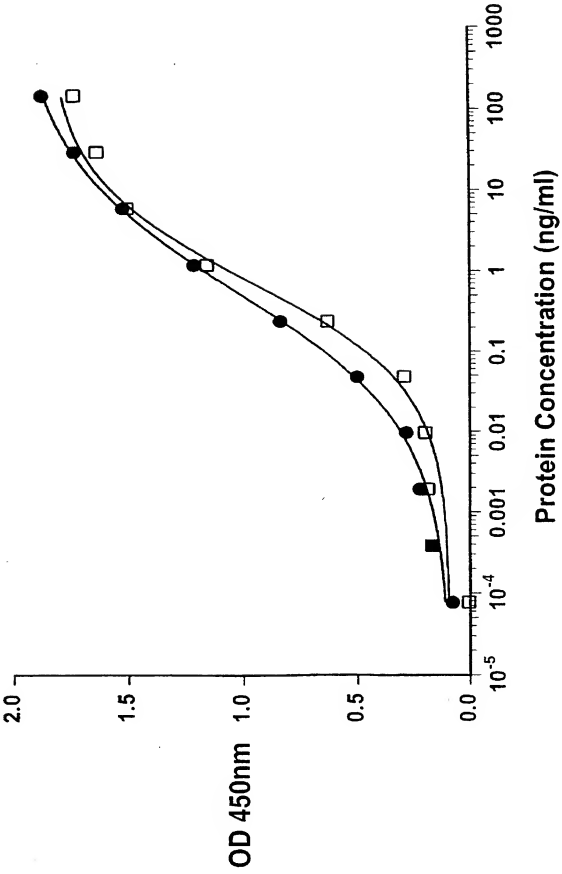

FIG. 3

Inhibition of neurotransmitter release from eSCN by LH_N/A and BoNT/A

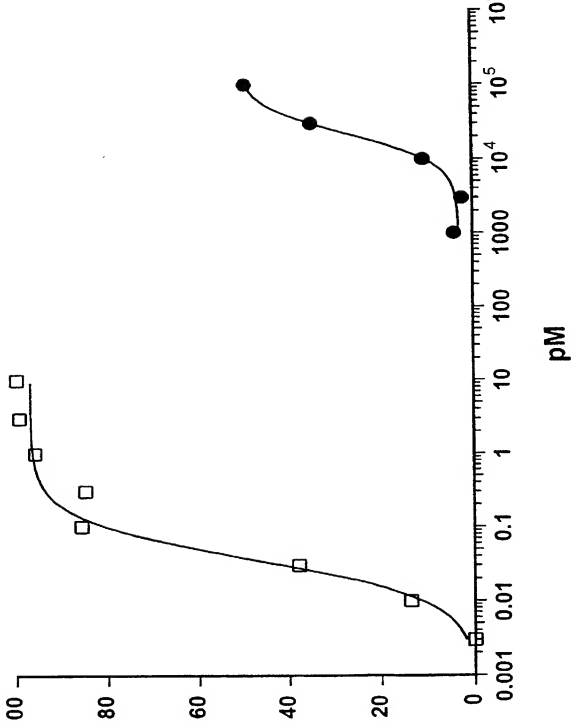

FIG. 4

Mouse toxicity data for purified vs. standard LH_N/A

	Mean number of survivors (/4)		
	50µg/mouse	20µg/mouse	5µg/mouse
Immunocolumn strategy (n=15)	1.60±0.41	3.67±0.16	ND
IMAC strategy (n=3)	1.33±0.88	3.33±0.33	ND
Mono Q® only (n=3)	0	0	1.33±1.33

10070764 .031202 21524

Combined Declaration for Patent Application and Power of Attorney with Delegation

Docket Number: ________________

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled Preparation of Highly Pure Toxin Fragments, the specification of which is attached hereto unless the following box is checked:

☐ was filed on ________________,
as United States Application Number or PCT International Application Number _________, and
was amended on __________________ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U S C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed	
9921592 3 (Application No)	United Kingdom (Country)	13/09/1999 (Day/Month/Year Filed)	☒ Yes	☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)	☐ Yes	☐ No

I hereby claim the benefit under 35 U S.C. § 119(e) of any United States provisional application(s) listed below

(Application No.)	(Filing Date)
(Application No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U S C § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

PCT/GB00/03519 (Application No)	13/09/2000 (Filing Date)	Pending (Status - patented, pending, abandoned)
(Application No)	(Filing Date)	(Status - patented, pending, abandoned)

Appl. No
Docket No.

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Robert G Sterne, Esq., Reg No 28,912; Edward J. Kessler, Esq , Reg No 25,688, Jorge A Goldstein, Esq , Reg. No. 29,021, Samuel L Fox, Esq., Reg No 30,353; David K S. Cornwell, Esq., Reg No 31,944, Robert W Esmond, Esq., Reg. No 32,893, Tracy-Gene G. Durkin, Esq , Reg No. 32,831; Michele A Cimbala, Esq., Reg No 33,851; Michael B. Ray, Esq., Reg No 33,997, Robert E. Sokohl, Esq., Reg No 36,013, Eric K. Steffe, Esq , Reg No 36,688; and Michael Q Lee, Esq., Reg No. 35,239.

I hereby authorize the U S attorneys named herein to accept and follow instructions from Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL, ENGLAND, as to any action to be taken in the U S Patent and Trademark Office regarding this application without direct communication between the U S attorneys and me. In the event of a change in the persons from whom instructions may be taken, the U S attorneys named herein will be so notified.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Appl No.
Docket No

3-00

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(Supply similar information and signature for subsequent joint inventors, if any)

POWER OF ATTORNEY FROM ASSIGNEE WITH DELEGATION

Microbiological Research Authority , having a principal place of business at CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG, Great Britain , is assignee of the entire right, title, and interest for the United States of America (as defined in 35 U S C §100), by reason of an Assignment to the Assignee executed on 4 & 27 FEB 2002 of an invention known as Preparation of Highly Pure Toxin Fragments (Attorney Docket No. _____ , which is disclosed and claimed in a patent application of the same title by the inventor(s) John Andrew Chaddock, Frances Celine Gail Alexander and Keith Alan Foster (said application filed on _____ at the U S. Patent and Trademark Office, having Application Number _____)

(13)

The Assignee hereby appoints the following U S attorneys to prosecute this application and any continuation, divisional, continuation-in-part, or reissue application thereof, and to transact all business in the U S Patent and Trademark Office connected therewith Robert Greene Sterne, Registration No. 28,912, Edward J. Kessler, Registration No 25,688; Jorge A Goldstein, Registration No. 29,021, Samuel L. Fox, Registration No 30,353; David K.S. Cornwell, Registration No. 31,944, Robert W Esmond, Registration No 32,893, Tracy-Gene G. Durkin, Registration No. 32,831; Michele A Cimbala, Registration No. 33,851, Michael B Ray, Registration No. 33,997, Robert E. Sokohl, Registration No 36,013; Eric K Steffe, Registration No 36,688, Michael Q Lee, Registration No 35,239; and Steven R. Ludwig, Registration No 36,203. The Assignee hereby grants said attorneys the power to insert on this Power of Attorney any further identification that may be necessary or desirable in order to comply with the rules of the U.S. Patent and Trademark Office.

The Assignee hereby authorizes the U.S attorneys named herein to accept and follow instructions from Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL, Great Britain as to any action to be taken in the U S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and the Assignee. In the event of a change in the persons from whom instructions may be taken, the U S attorneys named herein will be so notified by the Assignee.

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FOR Microbiological Research Authority

SIGNATURE

BY J M HANLON

TITLE SECRETARY MRA (CAMR)

DATE 28 FEBRUARY 2002